A Study of the Effect of Weightlessness on the Biochemical Response of a Monocotyledonous Seedling

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The characteristic fashion in which plants respond to gravity has been known for centuries. Yet the recognizable effects of gravity can be nullified in either a mature leafy plant or a germinating seedling merely by rotating it about its horizontal axis (Ruhland et al., 1962). When the Biosatellite program was conceived, however, it was not known whether plants grown in weightlessness would respond with epinastic curvatures and biochemical activities identical to those of plants grown on a clinostat on Earth.

The bulk of the evidence used to explain the mechanism governing the epinastic response of a plant to a gravity-nullified environment is based upon the translocation of auxin (Boysen-Jensen, 1936; Gillespie-Pickard and Thimann, 1966; and Lyon, 1962). Yet, the basic biochemical phenomena within the plant that accompany the movement of the hormone were and still are not known. A physiologic "penalty" results from biologic work in all living systems. It seems inconceivable, therefore, that the profound disorientation of the plant organs typical of growth on a clinostat could occur without a concomitant conservation or utilization of energy.

The experimental program was designed to: (1) Relate the anticipated response to metabolic and energetic reactions throughout the length of roots and shoots; (2) determine whether changes in metabolism which occur on the clinostat would also be found in space-grown seedlings; and (3) compare the biochemistry of epinastic curvature with that of geotropic curvatures. The experimental protocol called for measuring the activity of key enzymes associated with some of the pathways of intermediary metabolism and energetics. These enzymes were examined for structural integrity because of speculation over the possible weakening of the tertiary forces which hold protein molecules together in a characteristic highly ordered manner.

The present report describes the results of the extensive biochemical analyses performed on the space-grown seedlings, their corresponding Earth controls, and postflight verification experiments. It will be shown that growth of seedlings on the clinostat simulates growth in a weightless environment. However, the biochemical parameters measured indicate that only auxin-mediated reactions are simulated by growth on the horizontal clinostat. One enzyme, peroxidase, showed evidence of increased activity at or near the site of epinastic curvature. Another enzyme, transaminase, also shows this tendency, but the data are less consistent and therefore less conclusive. The increased activity was found to be physiologic and not due to structural changes in the enzyme while the seedling is in the weightless state. The kinetic studies indicate that spaceflight does not affect the affinity of an enzyme for its substrate.

EXPERIMENT DESIGN AND METHODS

The primary objective of this study is to correlate changes in metabolism and energetics with the reorientation of plant organs expected, and indeed

observed, during weightlessness. The biochemical studies, which constitute our major effort, utilized the same specimens and experimental hardware as those of our coinvestigators studying other aspects of the developing wheat seedling in the weightless environment (see papers by Gray and Edwards, p. 123, and by Lyon, p. 167). The experimental design required that 78 seeds be planted in the flight package and in each of the Earth controls. The presoaked seeds were grown in a specially designed holder (see p. 168) filled with fully wetted ground vermiculite which permitted the organs to grow unhampered in water-saturated air. By allowing the seedlings to grow in this unhindered fashion, it was possible to observe changes in root orientation as well as in shoot curvature. The protocol called for postflight photographing of all the seedlings in order to determine the extent of root displacement and coleoptile curvature before the seedlings were divided for subsequent biochemical analyses.

The enzymes chosen for study in this program were selected to represent the biosynthetic activity of several pathways of energetics and intermediary metabolism. Figure 1 illustrates the logic used for selecting the key enzymes which catalyze the energy-yielding reactions in germinating seedlings. The relationships between the selected enzymes and the various metabolic functions are given in figure 2.

Materials and Methods

The plants examined in this study were seedlings *Triticum vulgare,* Georgia strain 1123. The unsoaked seeds were weighed, and only those weighing from 38 to 39 mg were used for flight and Earth control experiments. Additional selection was made on the basis of appearance (no fractures, etc.). The seeds were then surface-sterilized in 0.05 percent $HgCl_2$, washed in running distilled water, and soaked for 3 hours in aerated distilled water at 95 F.

The presoaked seeds were then inserted into the rubber diaphragm of the nontoxic polycarbonate stalks containing thoroughly wetted ground vermiculite. Each seed was inserted about half-way through the hole burned in each cap and then water was injected into the air spaces of the core. Water loss was prevented by applying a lanolin beeswax mixture at the point of contact between the rubber diaphragm and the seed. The stalks were then placed in the experiment package; 2 ml

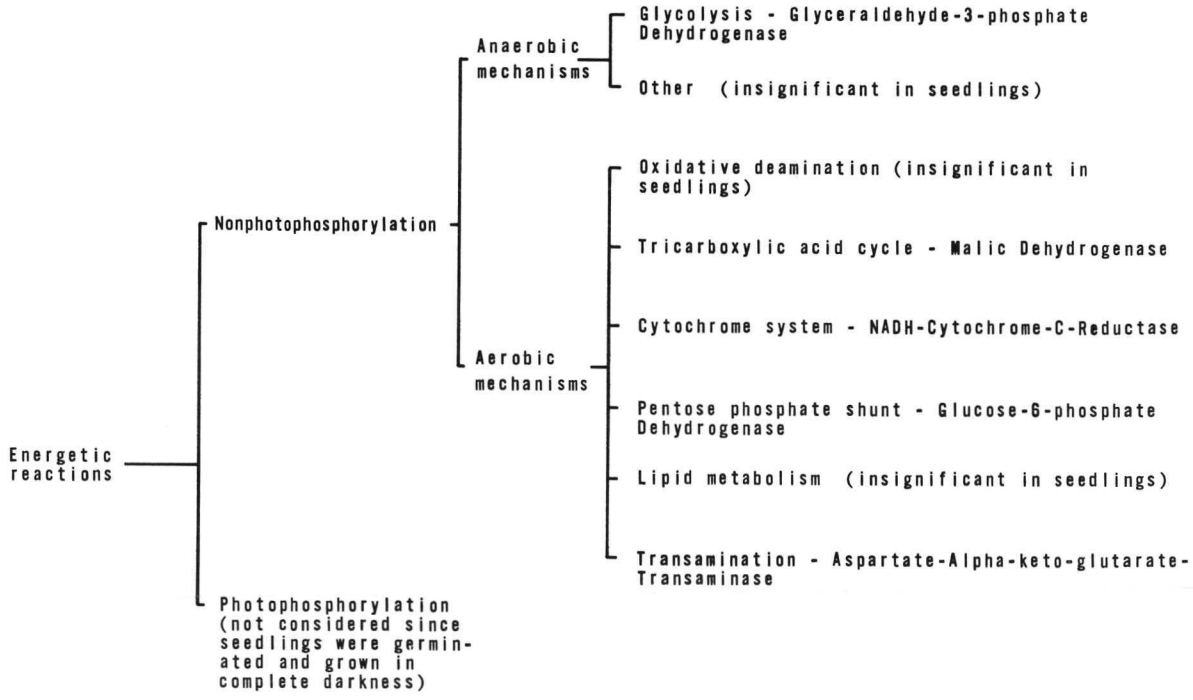

Energetic reactions
Nonphotophosphorylation
Photophosphorylation (not considered since seedlings were germinated and grown in complete darkness)
Anaerobic mechanisms
Aerobic mechanisms
Glycolysis - Glyceraldehyde-3-phosphate Dehydrogenase
Other (insignificant in seedlings)
Oxidative deamination (insignificant in seedlings)
Tricarboxylic acid cycle - Malic Dehydrogenase
Cytochrome system - NADH-Cytochrome-C-Reductase
Pentose phosphate shunt - Glucose-6-phosphate Dehydrogenase
Lipid metabolism (insignificant in seedlings)
Transamination - Aspartate-Alpha-keto-glutarate-Transaminase

FIGURE 1.— Correlation of enzymes with energy-yielding reactions.

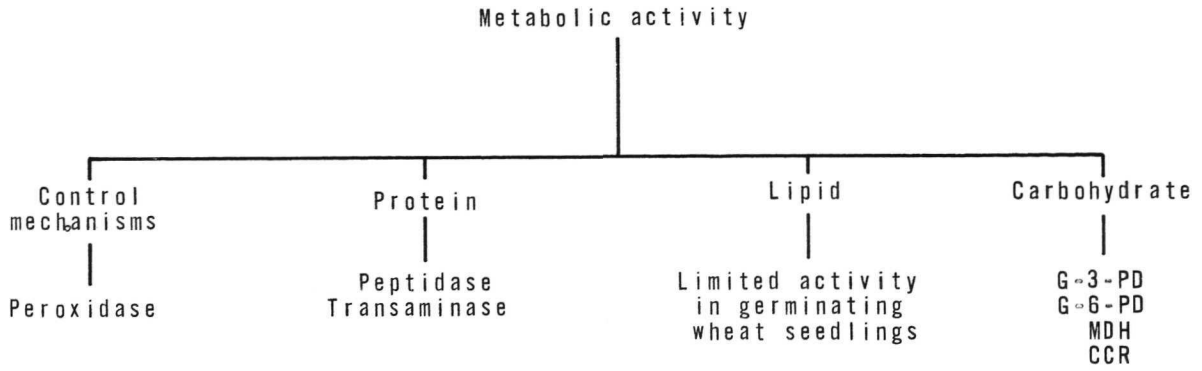


FIGURE 2.— Correlation of enzymes studied with metabolic functions.

of distilled water were added to each of the three small chambers and 5 ml, to the large chamber. Gas samples were taken and the lids were tightly sealed.

The biochemical studies were planned so that the information acquired could be directly correlated with the results of the morphologic and histologic investigations. The shoots were sectioned into 200-micron slices and the enzymatic analysis was performed on each slice. For convenience, meristematic mass was analyzed together with the shoot. Since the diameter of the root tissue is considerably less than that of the coleoptile, the primary root was sectioned into 1 mm slices to provide a sufficient concentration of enzymes for analysis. In the region of the root tip where enzymatic activity is greatest, the size of the slices was reduced to 0.5 mm in order to obtain more complete information. This procedure allowed a plot graph to be drawn which illustrates the enzymatic activity along the length of the tissue. The advantage of this particular approach is that the biochemical findings in each individual tissue can then be composed with its morphological response to orbital flight and, later, correlated with histologic and histochemical findings of similarly treated tissues. In this manner, any alteration in the physiologic processes resulting from weightlessness could be located within a confined area of the tissue and perhaps identified with a specific type of tissue or even a locus within the group of cells.

The unfixed seedling organs from the flight and Earth control packages were gathered by lifting the roots and the shoots as a unit from each seed. The roots were cut away and, for convenience, the scutella were left attached to the shoot. Each part was then inserted into a prepared agar slug, sealed with melted agar at 107.6 F, frozen first on Dry Ice to prevent cracking of the agar, and then preserved under liquid nitrogen until analyzed. The seedlings at several stages of processing are illustrated in figure 3. The organs from those seedlings fixed during the course of the experiment were treated differently. The organs were removed as a unit from the endosperm as described above, separated into three groups based upon organ length, and resuspended in FAA/DMSO fixative of the same formulation as that used for the original fixation without the organs being separated. These tissues were kept in fixative until they were

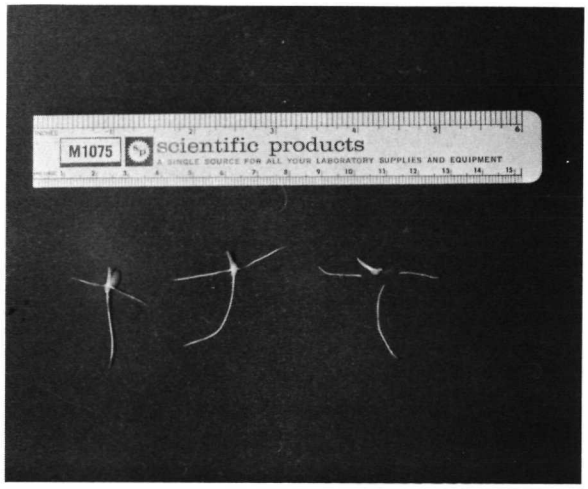


FIGURE 3.— Dissection of wheat seedlings in the first and last stages of harvesting. Roots and shoot removed as a unit from endosperm and roots trimmed away from shoot leaving the bulbous scutellum attached to the coleoptile.

analyzed. Confirmatory experiments were also performed on green leafy plants. For this purpose, immature 45-day-old pepper plants, *Capsicum annuum,* were grown in soil under continuous fluorescent daylight (Johnson and Tibbits, 1968).

Analytic Procedures

The gas content of each air sample was determined by standard gas chromatography techniques (Szymanski, 1964). The following enzymes were analyzed.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G–6–PD)

The analytical procedure employed for the studies of G–6–PD was a modification of the techniques described by Kornberg and Horecker (1962). Initial work established the pH optima for the reaction at 8.0, in 0.05 *M* Tris buffer, and the optimum substrate and cofactor concentration for the quantity of tissue present in the system. The activity in 200-micron slices of 65-hour coleoptiles and 1-mm slices of primary roots (except for the first 3 mm from the root tip where the slice size was reduced to 500 microns) was determined. At the conclusion of the analysis, each tissue was vacuum-dried at room temperature and weighed by using a Cahn RG Electrobalance.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (G–3–PD)

The procedure used for G–3–PD was a modification of that described by Racker (1965). Frozen tissue of 200-micron sections was used. At the conclusion of the assay, the sections were removed from the reaction vessel, placed on an aluminum pan, and dried under vacuum in a desiccator at room temperature for at least 6 days, and the dry weights were measured as before.

MALIC DEHYDROGENASE (MDH)

A modification of the method described by Siegel and England (1961) was used on each 200-micron section.

TRANSAMINASE

Transaminase was assayed according to the procedures described by Henry (1963) on each 200-micron slice of tissue.

CYTOCHROME C REDUCTASE (CCR)

Each tissue slice was added to 0.30 ml of a reaction mixture, at 32 F, comprised of 0.03 *M* Tris/Cl buffer at pH 7.85, 4.87×10^{-4} *M* DPNH, and 8.33×10^{-6} *M* cytochrome C. After each slice was added to the reaction mixture, the reaction flasks were incubated for 30 minutes at 98.6 F and then cooled again to 32 F, and the absorption was measured at 550 mμ and compared with that of suitable blanks.

PEROXIDASE

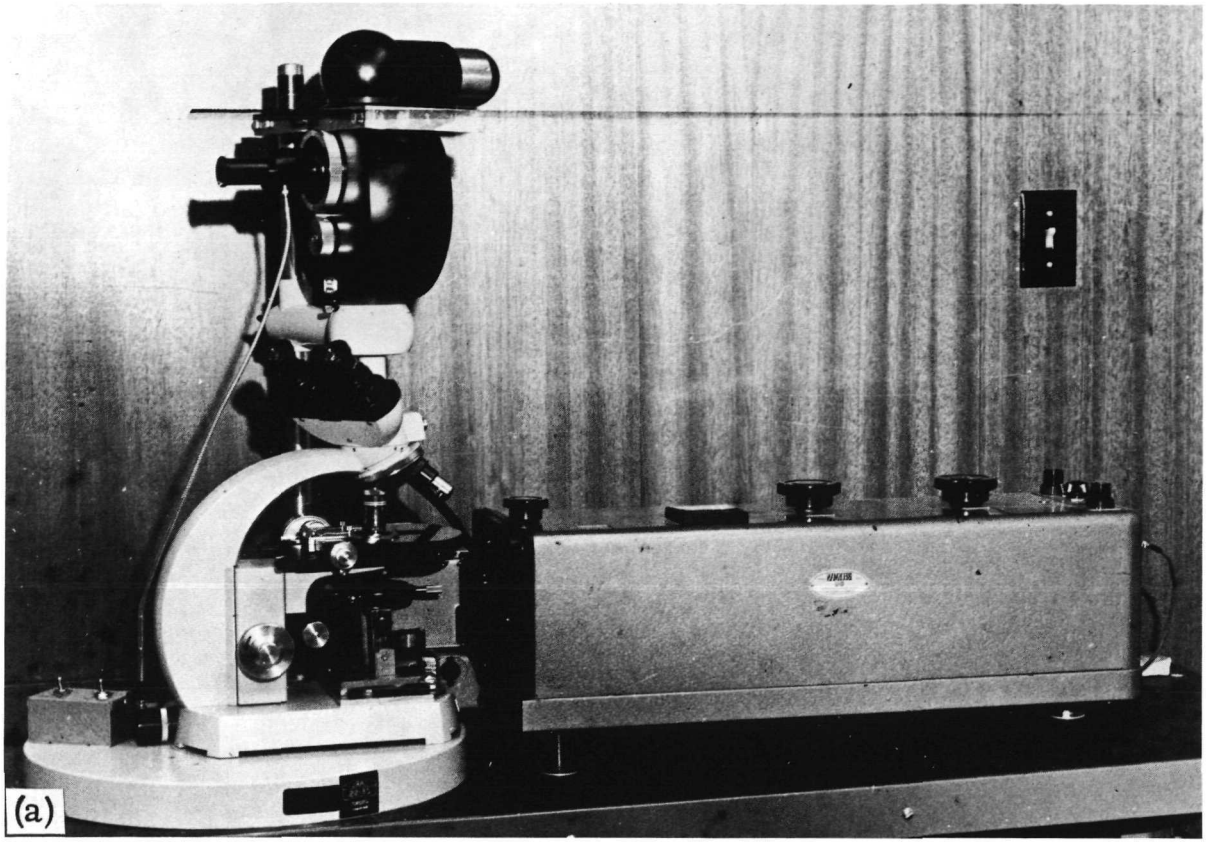
Peroxidase activity was measured in the homogenates by using a modification of the method described by Aurand et al. (1965). The determination is based upon the oxidation of a hydrogen donor (*p*-phenylenediamine dihydrochloride) in the presence of hydrogen peroxide to produce a colored compound. The shoots were sectioned while frozen into 200-micron slices. The protein concentration of each slice was determined spectrophotometrically according to procedures described by Murphy and Kies (1960).

The electrophoretic separation of peroxidase isozymes was performed in the following manner. A 2 percent agar solution was made by using 0.10 *M* borate buffer of pH 9.0. The hot solution was poured on a glass plate into 10- by 1-in. strips to a thickness of 3½ to 4 mm. The same buffer solution was poured into the electrode compartments of the electrophoresis cell. The tissue was dissected and the portions to be examined were placed on a 3- by 8-mm piece of filter paper on a glass plate. The tissue was crushed with a stirring rod and the tissue fragments were removed from the filter paper. Liquid-saturated filter paper was then inserted in slits cut into the agar slab.

A potential of 200 volts was applied for 90 minutes. After electrophoresis, the isozymes were visualized by flooding the agar surface with a solution that was 5×10^{-3} *M* in guaicol, 5×10^{-3} *M* in hydrogen peroxide, and 0.50 *M* phosphate buffer at pH 5.8.

PEPTIDASE

Peptidase activity was determined by using the titrimetric method of Linderstrom-Lang and Holter (1932). Each slice was incubated for 4 hours with alanylglycine at 104 F, and the liberated ammonia



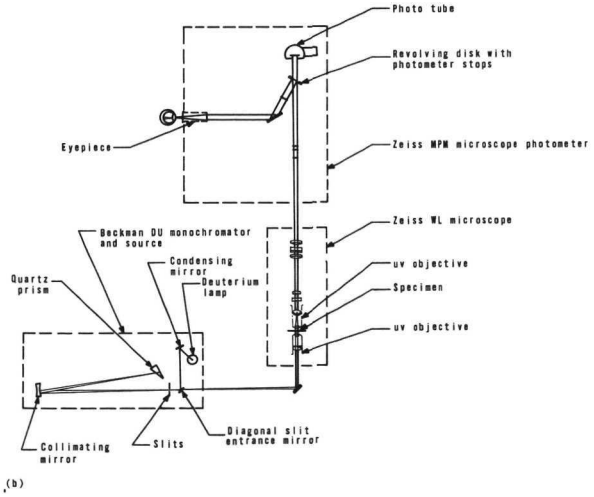


FIGURE 4.—Cytospectrophotometer. (*a*) Photograph. (*b*) Optical diagram.

was titrated with 0.05 *N* HCl. Each slice was then recovered and the dry weight was obtained.

Enzyme kinetics were employed in order to obtain Michaelis constants and the maximum reaction velocities. The calculations were made from plots of the reciprocals of velocity against substrate concentration, as described by Lineweaver and Burke (1934). The tissues used for the kinetic studies consisted only of lateral roots.

CYTOSPECTROSCOPY

Specimens for analysis with the cytospectrophotometer (fig. 4) were fixed with FAA and then dehydrated with a *t*-Butanol series and vacuum-infiltrated with a paraffin oil-paraffin series. The methods used were those of Johansen (1940). Sections 7 microns thick were floated on a bath with no adherent and picked up on clean, uncoated quartz slides. After they had dried for several hours the wax was removed and the tissue rehydrated with a xylene-ethanol-water series. A drop

of glycerine was placed on the section, which was then covered with a quartz cover slip. The cover slip was held in place by running a bead of paraffin around the edge.

The motor drive for the mechanical stage of the Zeiss Model WL microscope moved slides at a rate of 0.30 mm/min parallel to their length. Since comparison of the sides of the coleoptiles was desired, specimens were floated onto the slides with the coleoptile perpendicular to the length of the slide.

Before the adsorption was measured, the specimen was photographed with a low-power objective and a print was made. The specimen was then examined visually to determine a series of paths for measurement that appeared to be typical of the specimen and that avoided traces of paraffin, bubbles, and other contaminants. The stage was set to track one of these paths and the specimen was brought into focus with the high-power quartz objective. The field of view was moved off the specimen, the monochromator was set at the desired wavelength, either 260 mμ or 280 mμ, and the instrument was set to read 100 percent transmission. The recorder was then set for 0 and 100 percent transmission, and the mechanical stage drive and the recorder were started simultaneously. Care was taken to run a substantial distance before and after tracking across the specimen to insure that the portion of the slide was typical for 100 percent transmission for a given run.

After one or more runs were made on a given track, the wavelength was returned to the visible spectrum and the location of the track was plotted on the photograph of the specimen.

RESULTS

Growth and Morphology

The flight specimens appear to have grown normally. The average lengths of the roots and shoots under all conditions of growth are presented in table 1. No significant differences due to orbital flight were observed in organ length.

The orientation of the tissues of flight resembles that of those grown on the horizontal clinostat. Precise measurements of organ angles from the photographs of the seedlings recovered from space have already been reported (Lyon, 1968).

An apparent difference in the weight of meri-

TABLE 1. – Length of Organs of Seedlings From Both Flight of Biosatellite II and Earth Controls

Package	Coleoptile				Tap root				Lateral roots			
	65 hours		58 hours (fixed tissues)		65 hours		58 hours (fixed tissues)		65 hours		58 hours (fixed tissues)	
	Average length, mm	Standard deviation	Average length, mm	Standard deviation	Average length, mm	Standard deviation	Average length, mm	Standard deviation	Average length, mm	Standard deviation	Average length, mm	Standard deviation
Flight	4.14	0.90	2.78	1.18	21.54	6.73	7.12	4.05	10.04	6.76	2.04	1.96
Control I	3.44	1.13	2.52	.98	20.75	8.33	8.20	4.60	9.33	6.36	1.65	1.55
Control II[a], clinostat	3.30	1.39	3.30	.71	18.66	10.50	11.01	3.95	10.77	7.37	3.78	2.50
Control III, erect	3.76	1.15	2.72	1.00	23.98	8.09	9.37	5.32	13.29	6.13	3.64	3.53
Control III, clinostat	3.84	1.10	2.42	.84	20.38	6.71	6.53	3.97	10.02	5.99	1.43	1.47

[a] Tissue recovered at time of deorbit (60 hours).

stematic tissue between the space-grown seedlings and their comparable Earth controls was observed. This difference in the weight of this tissue was not found in either of the horizontal clinostat controls or the stationary erect Earth controls. In each instance, the dry weight of the meristematic tissue of the seedlings grown in space was less than that measured in corresponding Earth controls. These differences can be observed from the data presented in figure 5. No significant differences were found in slice weight along the shoot in the flight or Earth control specimens. Each curve represents the average of results for four specimens. These data compare favorably with protein measurements performed in a similar manner (fig. 6).

Epinastic Threshold

An area of particular interest to the wheat-seedling experimenters is the threshold of the epinastic response. The mission profile called for the spacecraft to provide 10^{-5} *g* for 95 percent of the flight. Extensive testing of the seedlings grown on a horizontal clinostat at an effective gravitational level of 10^{-5} *g* provided a high degree of confidence in the anticipated response of the seedlings to spaceflight. What would the response of the seedlings be if the craft failed to provide the desired *g* level?

In one study the effect of various continuous transverse accelerations below 1 *g* on the reorientation of roots and shoots of oat seedlings was measured (Dedolph, Gordon, and Oemick, 1966). The investigators inferred that there was no discrete threshold for gravity perception, although no accelerations between 10^{-2} and 0 *g* were studied. In a subsequent study (Shen-Miller, Hinchman, and Gordon, 1967) the threshold for georesponse to acceleration in gravity-compensated *Avena* seedlings was reported to be 1.4×10^{-3} *g* for shoots and 1.3×10^{-4} *g* for roots. The georesponses were measured between 65 and 70 hours after imposition of the centrifugal force.

A comprehensive investigation was conducted for measuring the onset of geosensitivity and

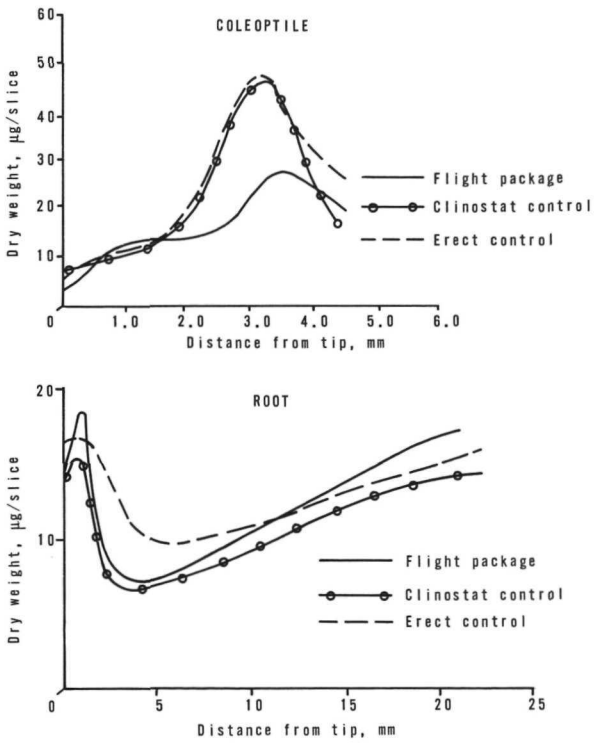


FIGURE 5.—Comparison of the dry weight of coleoptile and root sections of flight and Earth-bound control specimens.

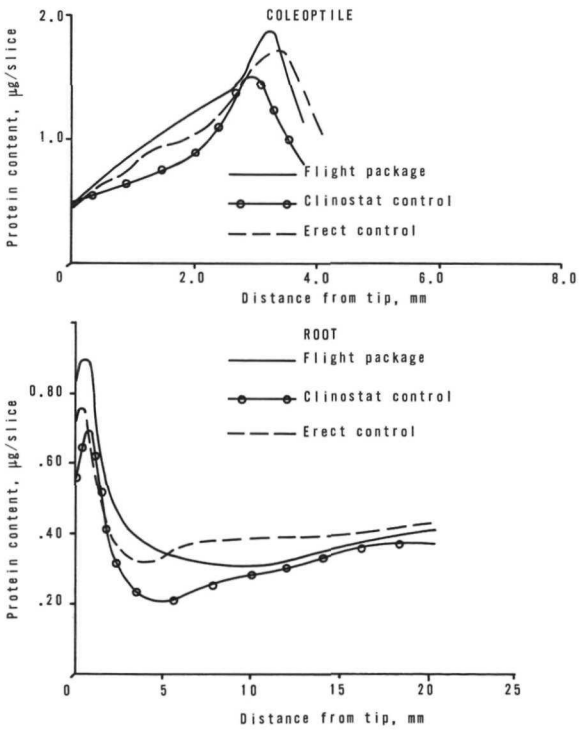


FIGURE 6.— Comparison of the protein content of coleoptile and root sections of flight and Earth control specimens.

thereby determining the threshold for epinasty in wheat seedlings. The *Avena* studies cited above were conducted with an experiment design differing from the protocol established by the wheat-seedling investigators. Thus, it was important to determine the epinastic threshold for the plants used in these experiments. Varying hypogravity environments were provided by adjusting the angle formed between the clinostat and the level bench top. Previous calculations (Ruhland, 1962) have shown that the sine of this angle is equivalent to the effective gravitational force experienced by the plants.

The results of this study (fig. 7) indicate that the onset of geosensitivity occurs at approximately 1/6 *g*. Capsicum and other green leafy plants, on the other hand, exhibit an epinastic threshold at approximately 2/3 *g* (fig. 8). These are well above the effective gravitational force predicted for, and indeed observed in, Biosatellite II. We can only conclude, therefore, that the response of the seedlings to growth in the orbiting spacecraft was within the sensitive range at all times. These studies have been amplified and reported elsewhere in greater detail (Conrad and Yokoyama, 1969 and 1970). The growth processes following deorbit until the tissues were dissected and frozen must be assumed to follow normal 1 *g* patterns.

Enzymatic Activity

The spatial distribution of enzymatic activities was determined in each of four separate shoots and roots from each condition of growth. No significant differences were revealed between the two sets of erect Earth controls (I and III–E) or the two sets of clinostat controls (II and III–C). Shoots ranging in length from 3 to 4 mm were selected for these analyses to minimize variations due to differing growth rates. Comparing data from one tissue with another appeared to be a difficult task because of the differences observed in organ length. Several shoots of different lengths were examined, therefore, in order to determine the enzyme distribution patterns. If a consistent pattern could be observed, it would then be possible to have a basis for comparison and thereby to obtain more meaningful results. Experiments were conducted in order to determine how enzyme distribution patterns differ in shoots of varying lengths. Peroxidase, G–6–PD, and G–3–PD activities were determined on shoots ranging from 3 to 18 mm in length. Initially the seedlings were grown in wrapped petri dishes on thoroughly moistened filter

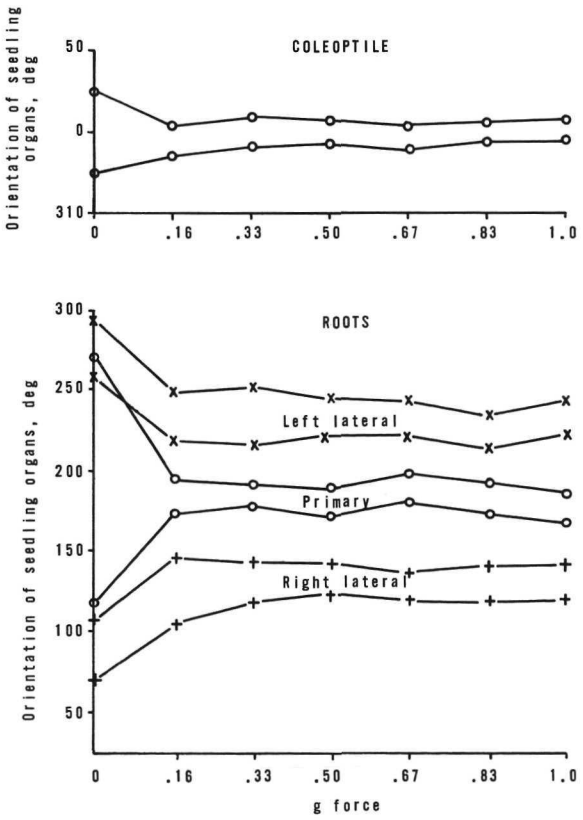


FIGURE 7.— Response of wheat seedling organs to a hypogravity gradient.

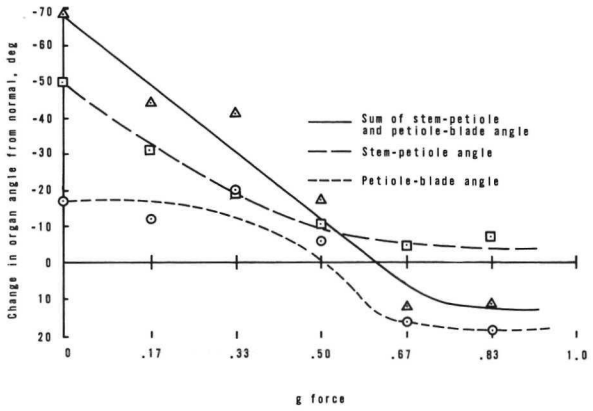


FIGURE 8.—Response of *Capsicum* to various levels of hypogravity.

paper. The findings were then confirmed by performing the same measurements on seedlings grown on the clinostat under simulated flight conditions. Proteins and dry weights were not measured, because our major effort was to relate enzyme activity to the kind of tissue examined.

This study indicates that the area of greatest enzymatic activity resides in the meristematic region (fig. 9). The activity along the shoot appears to remain relatively constant when the shoot length is 3 mm or longer. We now believe that meaningful comparisons between shoots can be made by identifying the meristematic region and adjusting the data to equivalent coleoptile length. Since there is little apparent difference in enzyme activity along the length of the shoot, adjusting the data to equal organ length is justifiable.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

The data illustrated in figure 10 indicate greater activity in the space-grown shoots than in the clinostat or erect-grown tissues. Although these differences appear to be relatively small, they are consistent. No differences in G–6–PD activity were found between the roots grown in space and those in the corresponding Earth controls.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

In figure 11, the specific activities of G–3–PD found in the space-grown shoots are slightly greater than those in comparably grown Earth controls. Values are averages for all tissue examined.

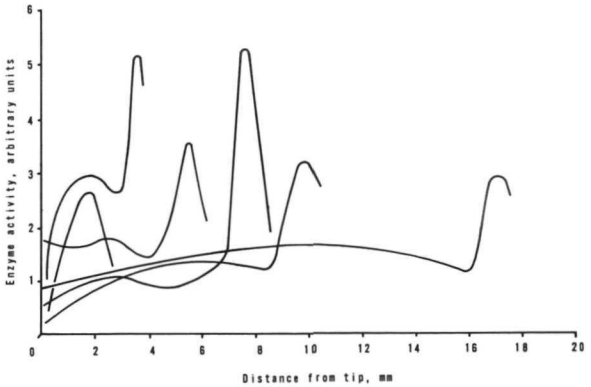


FIGURE 9.—Typical enzyme activity distribution in coleoptiles as a function of length.

MALIC ACID DEHYDROGENASE

The spatial distribution of MDH along the length of the shoots was determined (fig. 12). No significant alterations in the activity of this enzyme resulted from growth in a weightless environment.

CYTOCHROME C REDUCTASE

The analyses performed to date indicate no alteration in cytochrome C reductase activity due to weightlessness. The range of specific activities (fig. 13) falls well within the normal range of results found during our previous studies. As expected, the greatest relative activity was observed in the area containing the greatest bulk of tissue. The area containing greatest specific activity resides in the leaf primordium. No apparent differences could be observed between the two unfixed flight chambers.

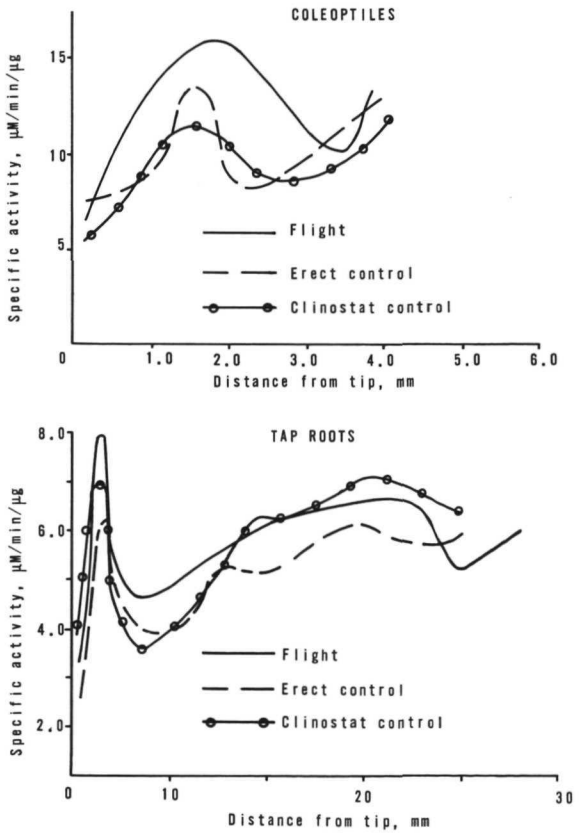


FIGURE 10.—Comparison of the specific activity of glucose-6-phosphate dehydrogenase in root and shoot sections from flight and Earth control specimens.

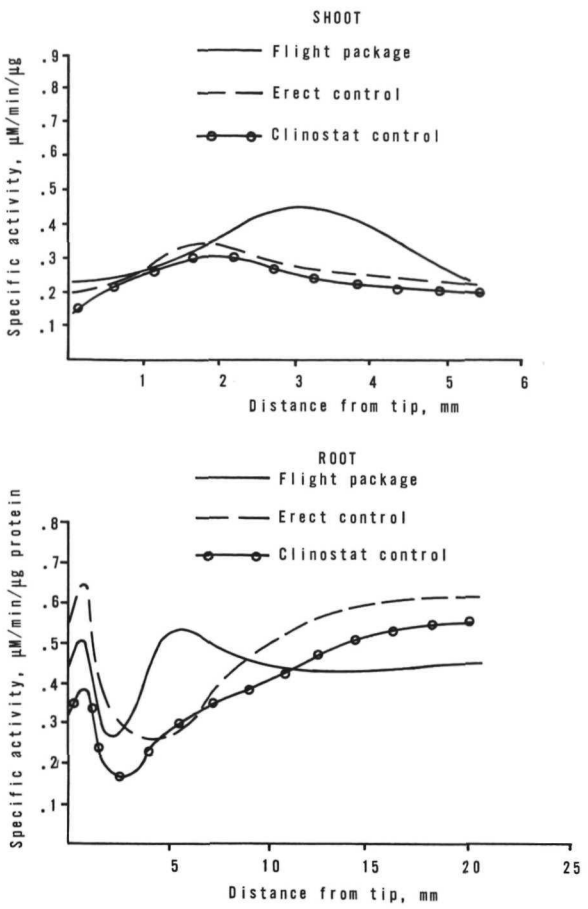


FIGURE 11.— Comparison of the specific activity of glyceraldehyde-3-phosphate dehydrogenase in root and shoot sections from flight and Earth control specimens.

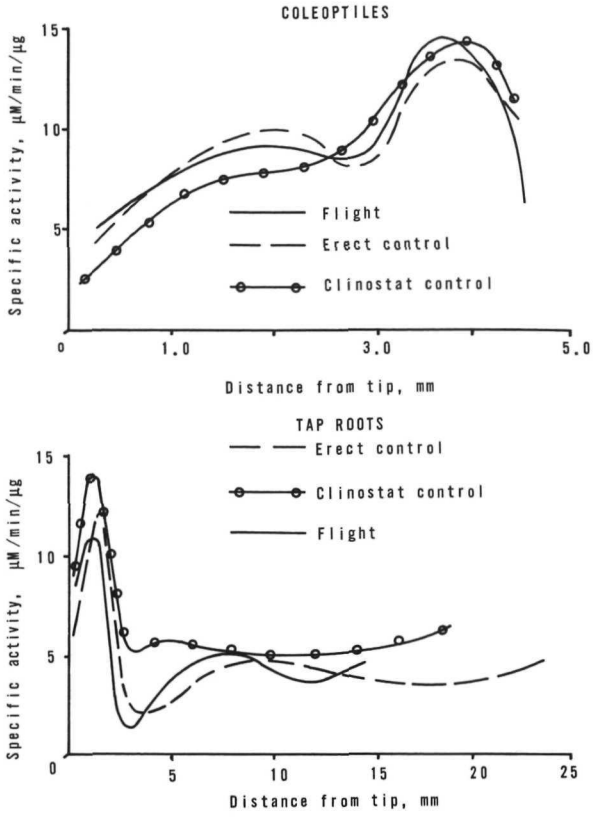


FIGURE 12.— Comparison of the specific activity of malic dehydrogenase in root and shoot sections from flight and Earth control specimens.

PEPTIDASE

The activity values of peptidase measured in the roots and shoots of the flight seedlings is comparable with the values obtained for the corresponding earth controls (fig. 14). No unusual patterns were found in any of the tissues set aside for the peptidase assay.

PEROXIDASE

The specific peroxidase activity is slightly higher near the site of epinastic curvature. This finding was observed in those tissues grown in weightlessness and on the horizontal clinostat. Erect earth controls not exhibiting epinasty show a relatively constant peroxidase activity. Furthermore, the increased activity appears to be related to the intensity of curvature (figs. 15 and 16) with greater activity found with more pronounced curvature. Subsequent attempts to determine whether the peroxidase activity varies between the convex and concave half of the curved tissue were inconclusive.

Since peroxidase activity appeared to be related to epinastic curvatures, it became necessary to perform two additional critical experiments. We wanted to learn whether peroxidase activity was affected preferentially on the concave or convex half of the curved tissue. We also thought it necessary to determine whether one particular isozyme was preferentially affected by gravity or whether each was equally affected.

Sectioning the primary root of 3-day-old wheat seedlings into two equal longitudinal halves proved to be impractical. Pea seedlings were grown therefore in the seed stalks in the same manner used for

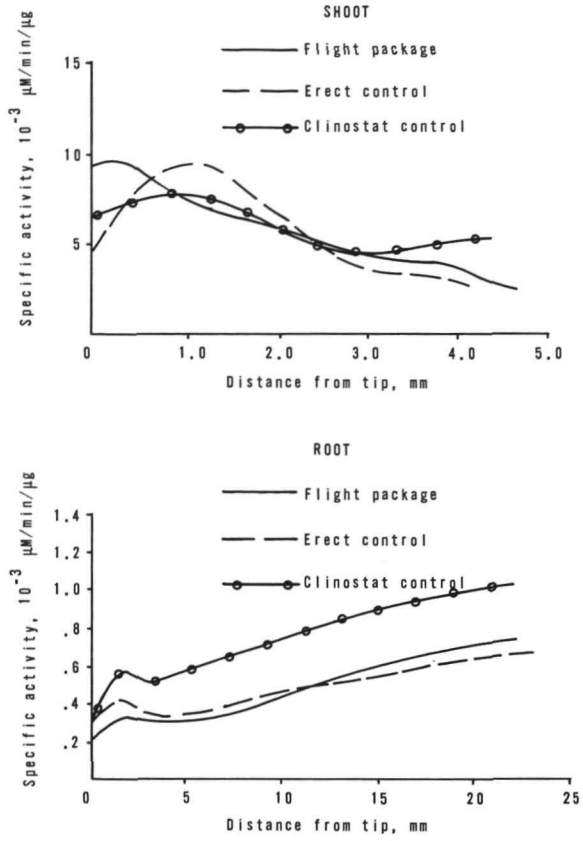


FIGURE 13.— Comparison of the specific activity of cytochrome C reductase in root and shoot sections from flight and Earth control specimens.

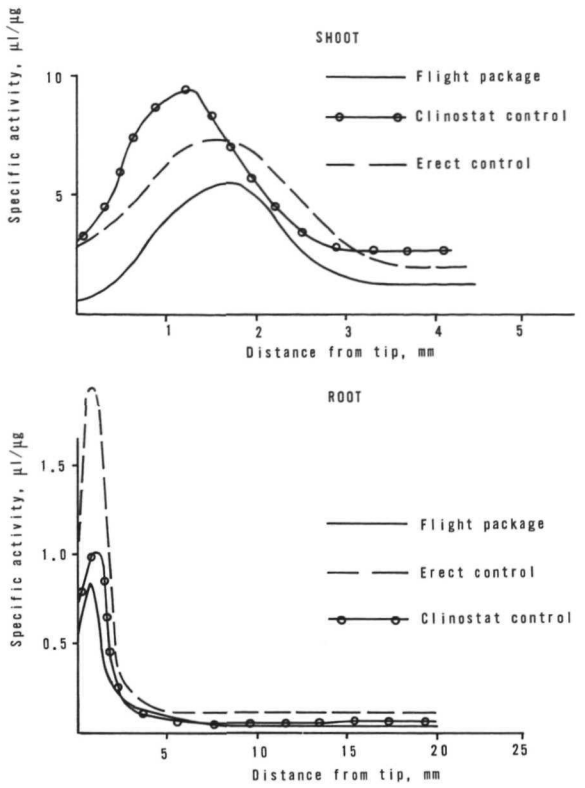


FIGURE 14.— Comparison of the specific activity of peptidase in root and shoot sections from flight and Earth control specimens.

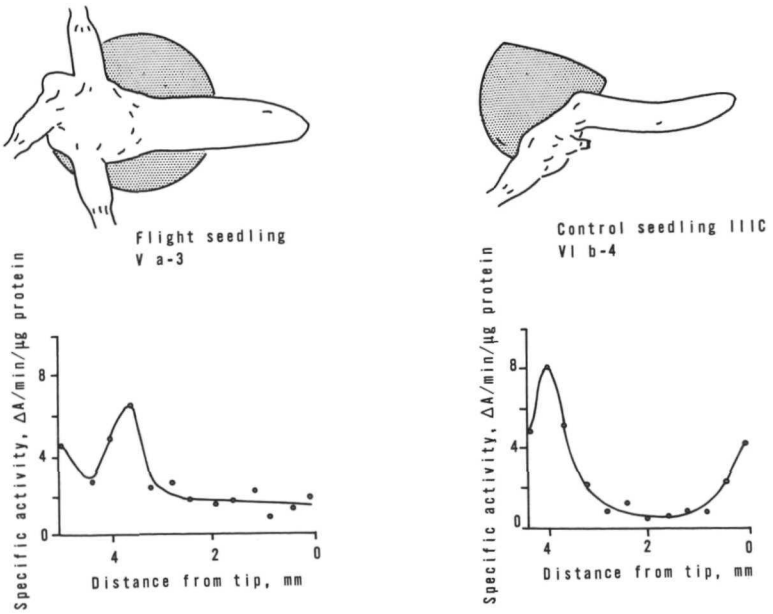


FIGURE 15.— The relationship of peroxidase activity to shoot curvature.

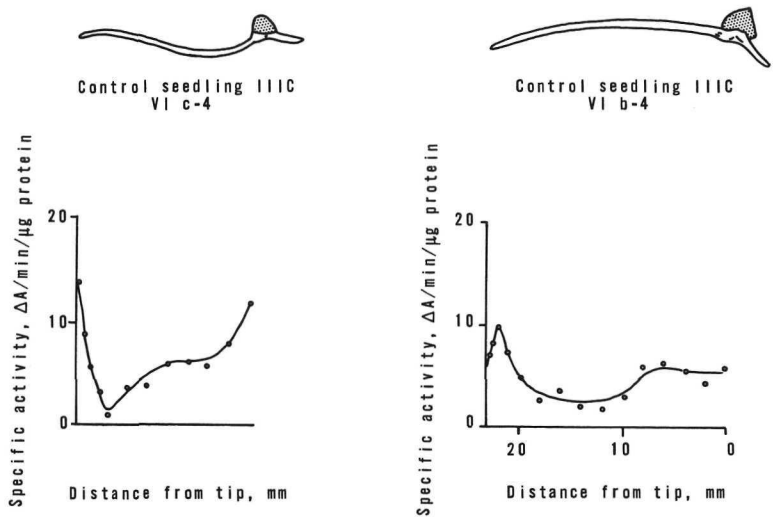


FIGURE 16.—The relationship of peroxidase activity to root curvature.

TABLE 2. – Peroxidase Activity in Convex and Concave Portions of Pea Roots

Growth conditions	Peroxidase activity o.d. 485/min/μg protein		Difference, %
	Convex	Concave	
Horizontal clinostat	12.7	11.1	14.4
Inverted, not rotated	9.2	9.1	Nil

wheat throughout the duration of this project. The pea root is considerably thicker, is easier to manipulate, and responds to rotation on a clinostat in the same manner as does the wheat. Consequently, all subsequent studies leading to the clarification of peroxidase involvement with gravity-dependent reactions in roots were performed upon pea root sections.

Phorograms of the peroxidase isozymes are presented in figure 17. It is quite evident that the techniques employed effectively separate the various isozymes of peroxidase. It is also clear that the root contains a greater number of isozymes and in more abundant quantities than do the coleoptiles. It is apparent that both light and gravity influence peroxidase activity. These phorograms confirm our decision to use peas for subsequent root studies, since the peroxidase isozymes appear to be similar in roots and shoots.

A comparison of total peroxidase activity between the convex and concave portions of both epinastic and geotropic curved tissues is given in table 2. A difference between geotropically induced curvatures and clinostat-induced curvatures was found. Peroxidase activity was found to be approximately 14 percent greater on the convex section of pea roots than on the concave or inner portion of the curved tissue. Geotropic curvatures induced by inverting the tissues did not show significant differences in peroxidase activity.

TRANSAMINASE

When transaminase was measured along the roots and shoots of space-grown and Earth controls, little difference could be found in the activities between specimens (fig. 18). We suspect, however, that some correlation between transaminase activity and curvature exists, but we have not accumulated sufficient evidence to form any definite conclusions.

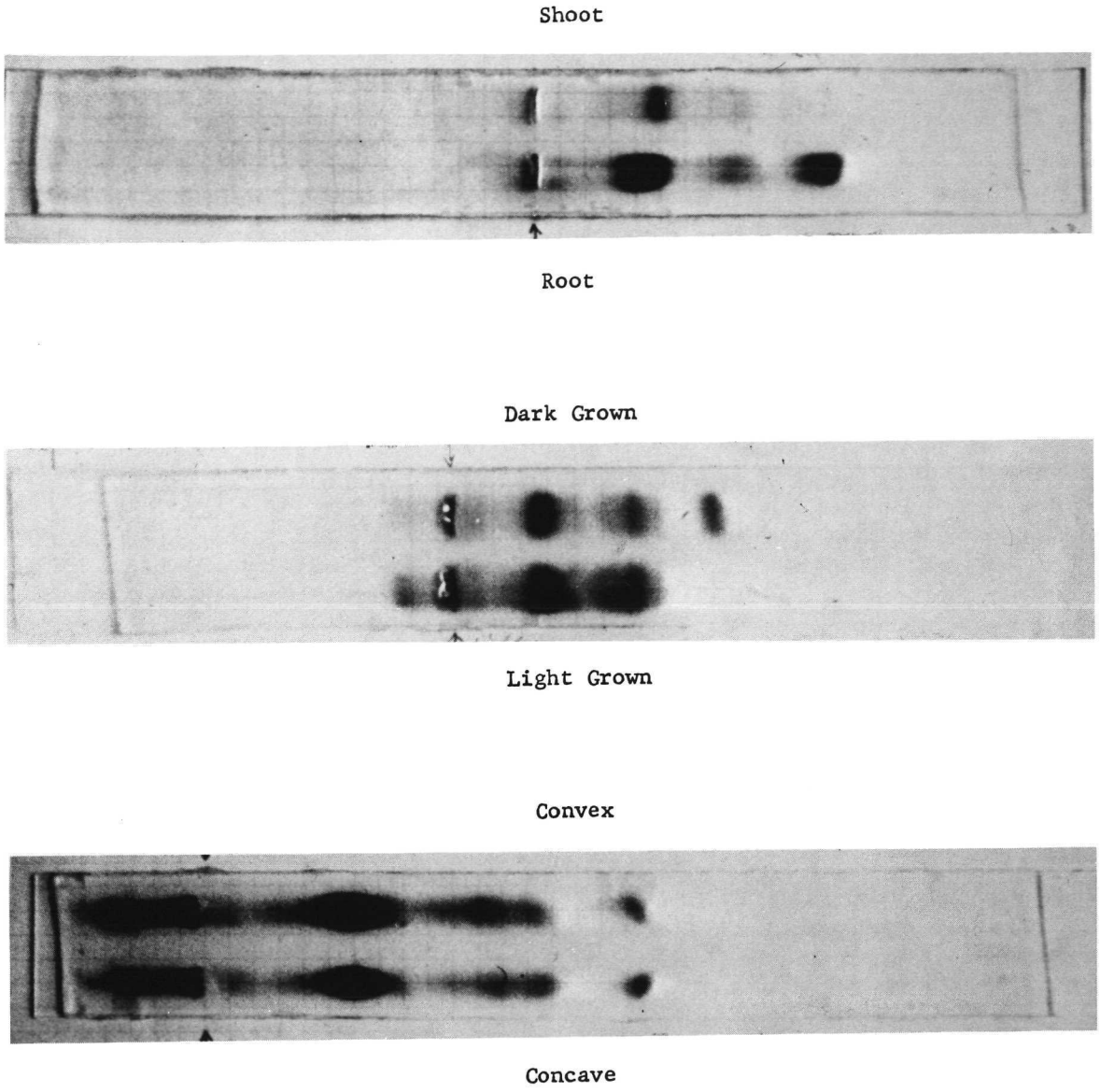


FIGURE 17.— Phorograms of peroxidase isozymes isolated from the roots and shoots of wheat.

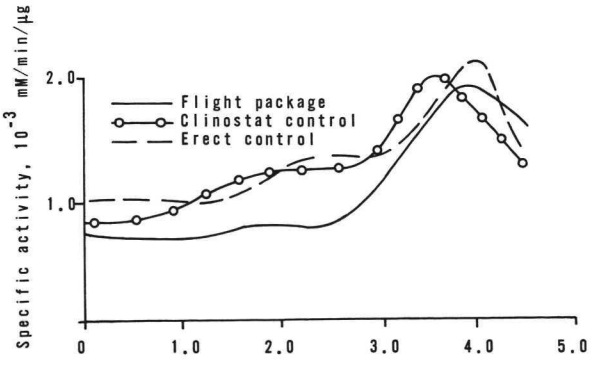


FIGURE 18.—Comparison of the specific activity of transaminase in coleoptile sections from flight and Earth control specimens.

TABLE 3. – Oxygen Measurement From Flight and Earth-Control Packages

Package	Oxygen, %		
	Before	After	Net change
Flight	20.7	20.9	+0.2
Control I, erect	20.8	20.8	None
Control II, clinostat	20.8	20.7	−.1
Control III–C, clinostat	20.7	20.5	−.2
Control III–E, erect	20.8		

Gas Analyses

RESPIRATION

The effects of spaceflight upon respiration of wheat seedlings were examined after the recovery of the spacecraft. Gas samples were obtained from the flight and from each of the control packages at the beginning and again at the conclusion of the experiment. Oxygen was measured by using the Sholander technique (Sholander, 1947). The analyses were performed for us by the Life Science Department of North American Rockwell Corp. From the data in table 3 we concluded originally that neither weightlessness nor the gravity-nullified environment provided by the horizontal clinostat affected respiration.

We now have reason to believe that our original conclusions might have been incorrect. Subsequent measurements (table 4) using the more sophisticated gas chromatography techniques at the Air Pollution Research Center at the University of California, Riverside, indicate that respiration is indeed influenced by gravity. Seedlings grown on the horizontal clinostat show evidence of increased respiration when compared with their comparably grown erect Earth controls. These new data compare favorably with those previously reported (Dedolph et al, 1966; Curtis, 1967).

ETHYLENE BIOSYNTHESIS

Several investigators have postulated the involvement of ethylene in the epinastic response. Small concentrations of ethylene in the air surrounding certain plants cause a triple response of (1) leaf epinasty, (2) stem swelling, and (3) inhibition of extensive growth of the 202 species and varieties tested; 89 showed ethylene-induced epinasty (Crocker et al., 1932). As little as 0.01 ppm of ethylene produced leaf epinasty in tomato plants. They also found that acetylene and propylene induced epinasty, but the concentration required to produce this response was 500 times greater than that required for ethylene. Our studies, using standard gas chromatographic techniques (Stephens et al., 1967), indicate that ethylene is not produced by wheat seedlings which germinate and grow in the dark (table 5). Ethylene, therefore, cannot be responsible for the root epinasty and shoot curvature exhibited by wheat seedlings under

TABLE 4. – The Effect of Light and Balanced Gravity on Respiration of Wheat Seedlings

Sample source	O_2, %	CO_2, %
Before growth	23.7	0.05
Light grown, rotated vertically	22.6	.78
Light grown, horizontal clinostat	21.9	1.53
Dark grown, horizontal clinostat	20.5	2.04

TABLE 5. – The Effect of Light and Balanced Gravity on Ethylene Biosynthesis and Utilization of Two Carbon Fragments

Sample source	Ethylene, ppb	Ethane, ppb	Acetylene, ppb
Before growth	56.0	232.0	59.0
Light grown, rotated vertically	79.0	236.0	58.1
Light grown, horizontal clinostat	100.0	158.0	32.5
Dark grown, horizontal clinostat	53.0	99.5	30.0

the gravity-nullified conditions of our experiment. In the presence of light, however, ethylene biosynthesis is stimulated and the epinasty of plant organs becomes more pronounced.

Cytospectrophotometry

The fixed seedlings from the flight experiment and the postflight controls were examined cytospectrophotometrically. Each slide was photographed and enlarged prints were made. The area scanned was then recorded on the photograph for future reference. An example of the recording technique is provided in figure 19. The results of a typical analysis of flight, erect, clinostat, and inverted coleoptiles are presented in figures 20 to 23. It is apparent that the fixed seedlings were too young to exhibit differential growth rates in the coleoptiles recovered from the flight or their corresponding Earth controls. The major finding as a result of this analytic technique is that when growing seedlings are inverted and geotropic curvatures are induced in the coleoptile the growth rate of the cells in the concave portion is reduced whereas the growth rate in the cells of the convex half continues to increase at a normal rate.

Postflight Controls

Extensive postflights studies were conducted in order to establish whether the physiologic alterations observed in tissues grown in space were caused by balanced gravity or such other physical factors associated with spaceflight as vibration, acceleration, and shock. These tests included growing wheat seedlings in the same experiment hardware used during Biosatellite II and examining the effect of vibration and acceleration upon the same physiologic parameters measured in the tissues recovered from Biosatellite II. The purpose of this exercise was to factor out those physical conditions, other than weightlessness, which were responsible for the physiologic aberrations observed in the wheat seedlings grown in space.

With the possible exception of G–6–PD, vibration did not appear to influence any of the biochemical parameters measured. Neither respiration, enzyme activity, nor growth appeared to be significantly different in those seedlings which were vibrated at Biosatellite II levels when compared with equivalent nonvibrated controls. It is quite evident from these studies that the reorientation of seedling organs, with the concomitant biochemical and physiologic aberrations observed in the tissues recovered from space, was the result of balanced gravity (weightlessness) and not due to the accompanying physical factors.

DISCUSSION

The conditions for growth in this experiment were unique. The seeds remained on the Earth for the first 15 hours of the experiment. During this time imbibition of water occurred. The seeds were then subject to approximately 45 hours of weightlessness, during which time imbibition presumably continued and growth of the roots and

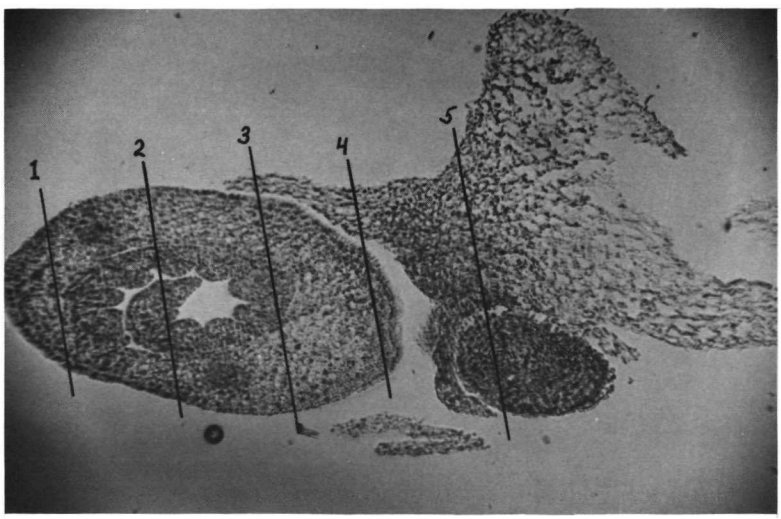


FIGURE 19.— Typical series of cytospectrophotometric scans through the coleoptile and scutella of a fixed seedling.

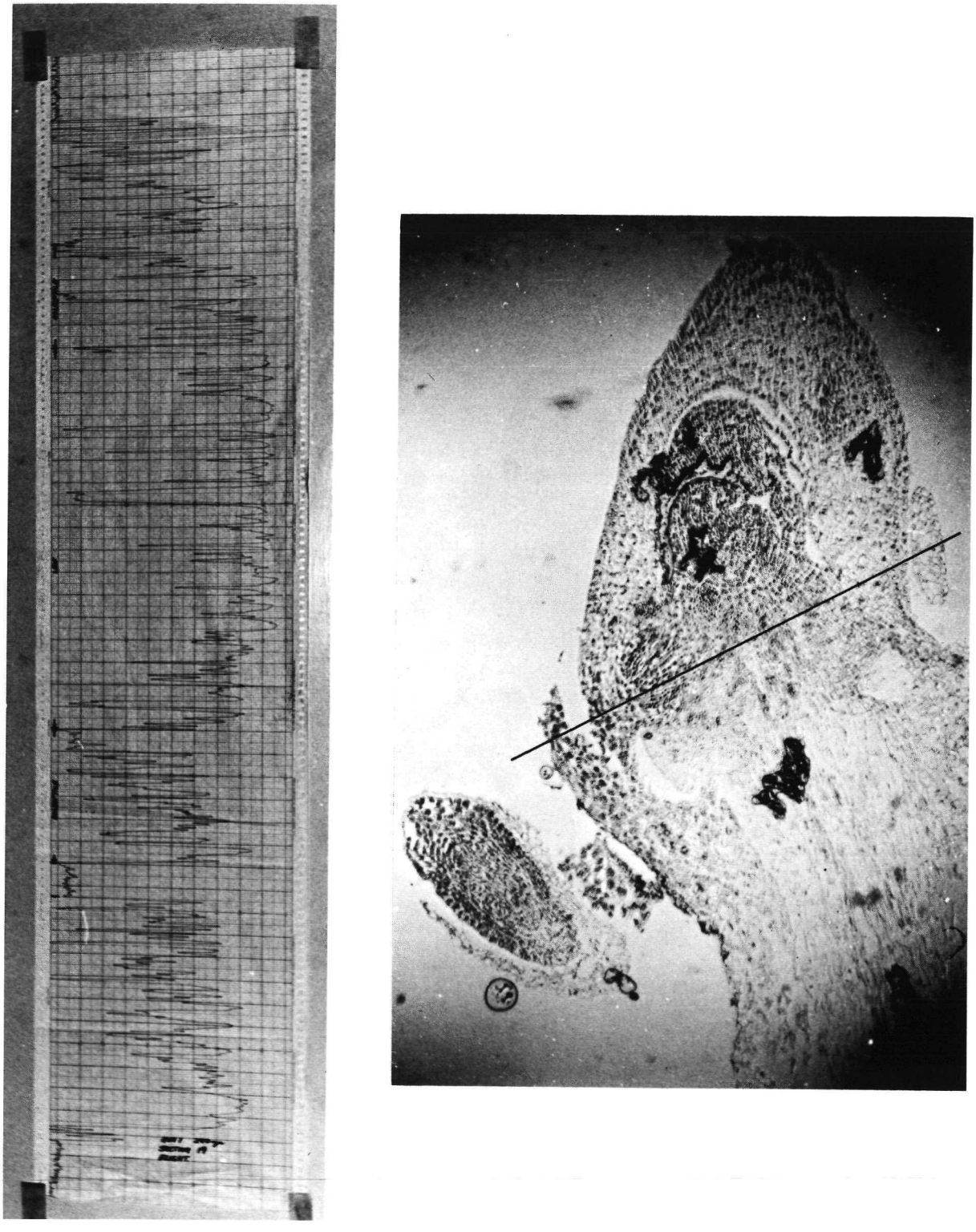


FIGURE 20.— Typical cytospectrophotometric scan of a fixed flight tissue.

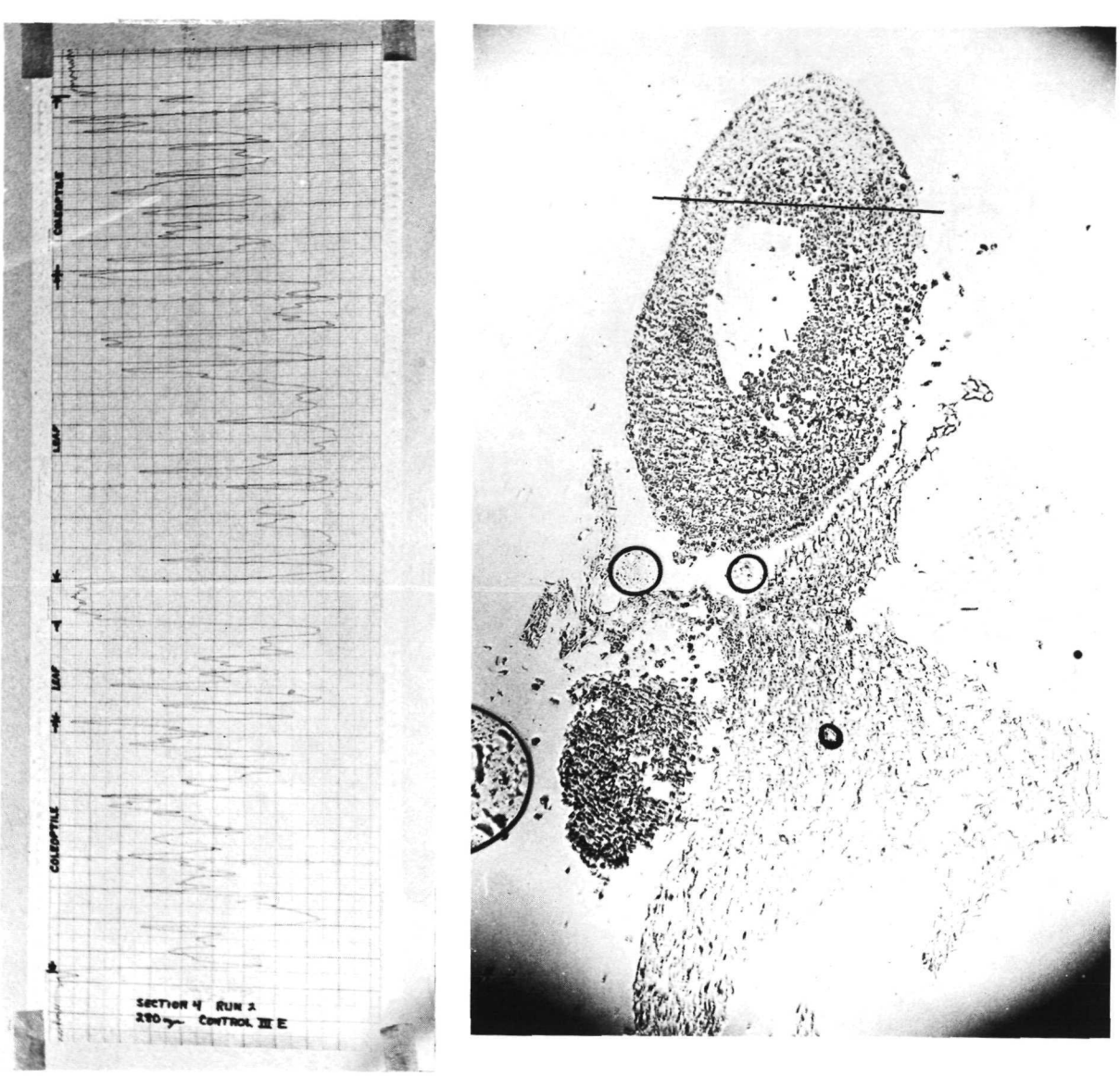


FIGURE 21.— Typical cytospectrophotometric scan of a fixed erect Earth control tissue.

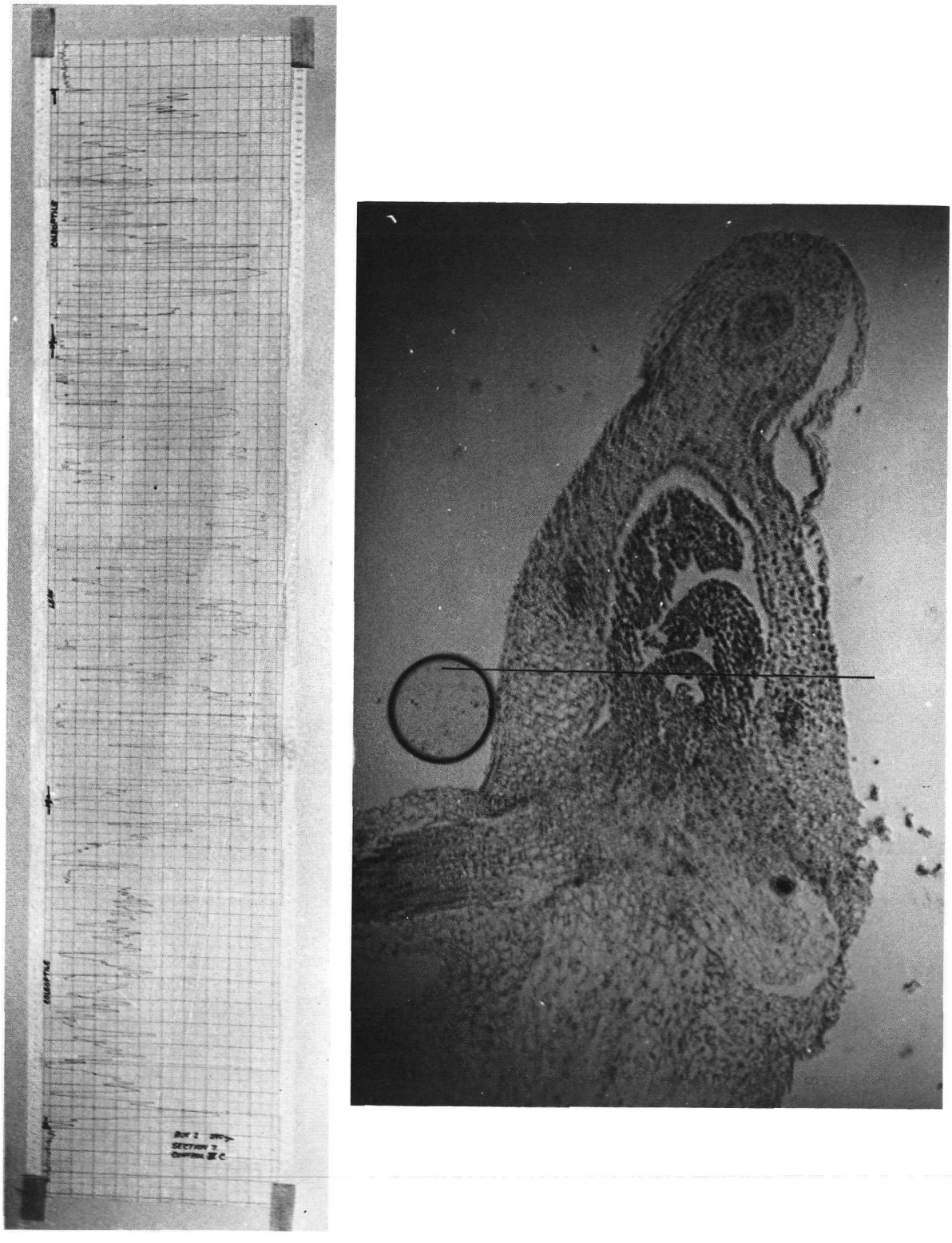


FIGURE 22.— Typical cytospectrophotometric scan of a fixed clinostat control tissue.

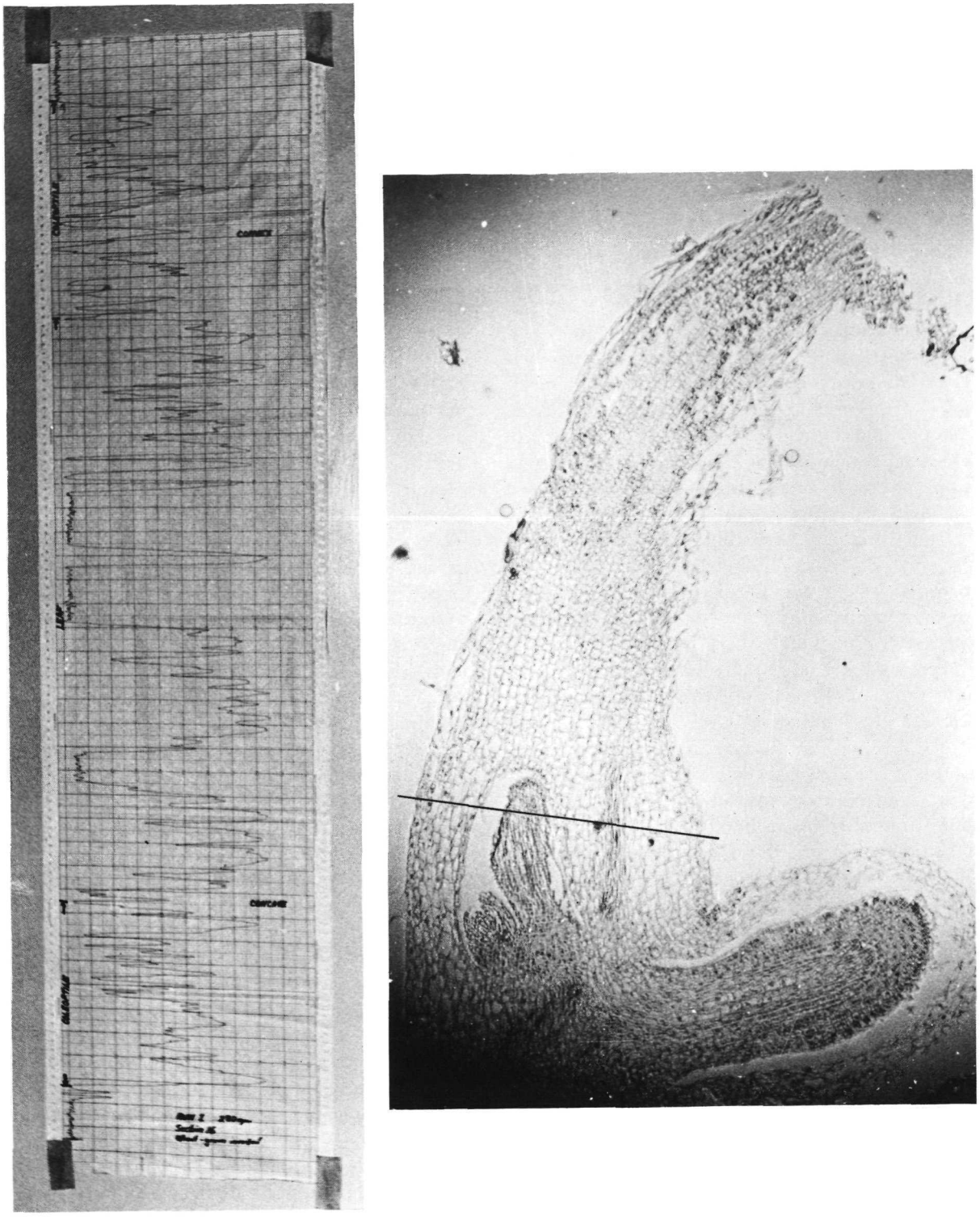


FIGURE 23.— Typical cytospectrophotometric scan of a fixed inverted tissue in which geotropic curvature was induced.

shoots was evident. A number of the specimens were fixed in space after approximately 42.5 hours of weightlessness, or about 58 hours of elapsed experiment time. The remainder of the seedlings grew for a total of roughly 44.5 hours in weightlessness. Growth continued for an additional 5 hours at 1 *g* before the package was opened for inspection and analyses.

It appears from the data presented in table 1 that the germination and growth of the wheat seedlings were normal during the mission of Biosatellite II. There does not appear to be any significant difference in the length of the roots and shoots between seedlings flown in space and their Earth controls. The overall growth and appearance of the seedlings grown in the space environment or under the gravity-nullified conditions provided on the horizontal clinostat are no different from the growth and appearance of seedlings grown on erect stalks normal to Earth's gravity. The normal variability in seedling growth far exceeds the small differences in organ lengths observed between the flight and Earth control seedlings and, thereby, contraindicates the temptation to report altered growth rates.

Profound effects were observed, however, in the orientation of seedling organs grown aboard Biosatellite II. The lateral roots grew, in the main, in the first and fourth quadrant rather than in the normally occurring second and third quadrants. The primary root, instead of growing straight down, appeared to grow off at an oblique angle. The orientation and displacement of the roots and shoots of the seedlings grown in space were similar to those obtained only from growth on a horizontal clinostat under similar conditions of time and temperature. From visual inspection alone, it was not possible to distinguish the weightless seedlings from their comparable Earth controls. The gross morphology of the seedlings grown in space is similar to that obtained on the horizontal clinostat (Lyon and Yokoyama, 1966).

The description which best characterizes seedling growth in space is that the roots grow in a manner more random than is normal. On Earth, root growth is highly ordered and occurs along a regular path. In space and on the clinostat, however, the highly ordered direction of root growth is replaced with increased randomness. This may be attributed to the loss of normal auxin control during weightlessness, but the biochemical reactions associated with such auxin control remain unsettled. Biochemical measurements performed upon the seedlings recovered from orbital flight indicate that metabolic changes are also associated with growth in a weightless environment. The metabolic functions affected appear to be respiration, carbohydrate metabolism, and an as yet unidentified mechanism in which peroxidase is intimately involved.

Although oxygen and carbon dioxide measurements of the air in the plant growth chambers indicated no changes in the respiration of the space-grown or Earth control seedlings (table 2), new evidence has been acquired which questions that original observation. The titrimetric method of analysis originally used was found to be less accurate than the gas chromatographic methods used in the carefully controlled postflight experiments. It now appears that seedling growth on a clinostat results in increased respiration (table 4). These newer findings are in close agreement with earlier published reports by other investigators (Dedolph et al., 1966; Curtis, 1967). Since the overall physiology of the seedlings grown on the clinostat resembles that of space-grown seedlings, it is likely that respiration did increase during weightless growth. This conclusion can only be confirmed through subsequent space experiments.

Carbohydrate metabolism also appeared to have been affected in the wheat seedlings grown during the flight of Biosatellite II. Glucose-6-phosphate dehydrogenase appeared more active per unit weight in the shoots of orbited plants than in the shoots of the others. This difference occurred in the coleoptile rather than in the leaf (Conrad, 1968). Furthermore, this one area stands out as the only example of a metabolic difference between space-grown and clinostat-grown seedlings. A critical examination of the tissues from the postflight controls indicates that the vibration profile may have increased the activity of this enzyme in vivo. If vibration and clinostat data are grouped together, the enzyme activity appears to differ from that of the stationary erect controls. Since Gray and Edwards (1969) reported cytologic similarities between clinostat and vibrated seedlings, the rationale for grouping the data in this fashion may be valid. No conclusive evidence has been forthcoming despite the large number of analyses performed on this enzyme. Because of the importance

of this enzyme in the growth processes of all living creatures, the effects of weightlessness on this enzyme should be critically examined on tissues from future space experiments.

Extrapolation of these data indicates that the energy for plant epinasty could be generated through altered carbohydrate metabolism. The increase in G–6–PD activity, together with increased respiration, suggests such a possibility. This relationship, if substantiated, carries enormous implications to all life in space, since almost all forms of life, both in the plant and animal kingdoms, respire in a uniform manner.

The effect of spaceflight on glyceraldehyde-3-phosphate dehydrogenase activity is more ambiguous. Activity measurements in the organs of seedlings grown in the large chamber indicate greater G–3–PD activity than in those of the seedlings grown in the small chamber. Although this finding occurred in each of the two seedlings examined, the total number of seeds taken from each chamber was small and the results may have been fortuitous. There appears to be no explanation capable of withstanding severe scrutiny which can establish the role of chamber size (within the limits of the experiment) to the growth processes. Since the differences in activity observed represent the extreme values within the range of variability of a large number of tissues, it must be concluded that spaceflight does not affect the reaction catalyzed by this enzyme.

Malic acid dehydrogenase, cytochrome C reductase, and peptidase activities were normal in all of the tissues examined. No differences were observed in the activity of these enzymes between the orbited and Earth control seedlings. Earlier ambiguities, particularly with MDH, have been clarified through additional analyses. Although the curve for the erect Earth control is now modified from the preliminary report (Conrad, 1968), the conclusion remains unchanged that there is no peculiar effect of weightlessness or the balanced gravity of the horizontal clinostat upon any of these enzymes.

The one enzyme most strikingly influenced during growth in a hypogravity environment is peroxidase. The activity of this enzyme increases dramatically in the region of epinastic curvature whether produced through growth on orbit or on the horizontal clinostat (fig. 11). No peaks of activity were found, however, in tissues which did not demonstrate epinasty (fig. 12). Transaminase activity also appears to follow this pattern, but the variability of this response dictates further study before a proper conclusion can be made. We have shown that peroxidase activity is stimulated under weightlessness. Although the exact biologic function of this versatile enzyme is not known (Saunders et al., 1964; Galston and Davis, 1969), morphogenic roles are suggested by its action in producing auxin (Riddle and Mazelis, 1964) as well as in activating auxin (Galston et al., 1953), in converting hydroxyphenylpropanes, such as coniferyl alcohol, to ligninlike materials (Siegel, 1955), and also in oxidizing such important metabolic compounds as NAD and NADP (Akazawa and Conn, 1958).

When normal genotypes are stunted by compounds which inhibit gibberellin biosynthesis, the peroxidase activity rises markedly (Gaspar and Lacoppe, 1968). The application of exogenous gibberellic acid depresses peroxidase synthesis and speeds growth, whereas gibberellin antagonists stimulate peroxide synthesis and retard growth. The evaluation of peroxidase in gibberellin-deficient plants is apparently helpful in oxidizing exogenous auxin. Ethylene production is stimulated by auxin, and ethylene causes an increase in peroxidase activity as well (Hall and Morgan, 1964). Auxin represses and kinetin promotes the synthesis of peroxidase in geranium (Galston et al., 1969). It appears, therefore, that these two growth factors also interact in the control of peroxidase activity as they do in the control of growth. Thus it appears that four different plant growth factors, auxin, gibberellin, cytokinin, and ethylene, can control the level of peroxidase activity in different tissues; and through a peroxidase-mediated mechanism they can cause the plant to react to reduced gravity with typical epinastic curvatures.

It has also been proposed that the response of pea roots to gravity is due to the ethylene production and, therefore, to growth inhibition on the lower sides of the roots caused by concentrations of auxin greater than the optimum for growth promotion (Chadwick and Burg, 1967). While it is evident that, as in stems, applied auxin elicits ethylene production and that this gives a growth inhibition, the theory has been criticized on the basis that growth responses in pea roots caused by supraoptimal auxin concentrations are different from those caused by ethylene (Andreae et al.,

1968). Auxin appears to reduce growth more rapidly than does ethylene. The auxin effect is reversible whereas that caused by ethylene is not, and even in the presence of ethylene the addition of high concentrations of auxin (10 to 200 micromoles per liter) further inhibits growth. The enhanced ethylene production might be contributory to the auxin inhibition of root growth (calculations indicated that 20 to 30 percent of the inhibition could be caused by ethylene), but it is evident that in roots, as in coleoptiles, the growth inhibition by high concentrations of auxin cannot be entirely explained by ethylene. This opinion (Galson and Davies, 1969) appears to have been confirmed by data returned from the Biosatellite II experiments, since no ethylene (0.1 ppb or less) was found in the wheat seedling growth chambers (Conrad, 1968).

These experiments have always been conducted in complete darkness in order to separate geotropic reactions from phototropic responses, both of which are controlled by auxin. If light is required for the biosynthesis of ethylene, it would be unlikely that this compound contributes to epinasty. Additional experiments were conducted, therefore, to determine the effect of light upon epinasty and ethylene biosynthesis in seedlings grown on the horizontal clinostat. The results of this study (table 5) indicate that ethylene biosynthesis is indeed a light-requiring reaction. Moreover, ethylene biosynthesis increases significantly when the seedlings are grown on a horizontal clinostat in the light. It has already been shown that ethylene interferes with the transport of auxin in seedlings as well as in leafy plants. It would also follow that epinasty in seedlings would be exaggerated by growth on a clinostat in the presence of light. Leafy plants, on the other hand, should respond to rotation on a clinostat in the dark with reduced epinasty.

This hypothesis was confirmed by experimental observations. Further reduction in epinasty occurs in 45-day-old pepper plants by preconditioning the plants to complete darkness for 24 to 48 hours. These experiments have been described elsewhere (Conrad and Yokoyama, 1970) and later confirmed by Lyon (1970). It appears therefore, that ethylene might have been partially responsible for the epinasty observed in the pepper plant experiment but not in the wheat seedling experiment. If the controlling mechanism functions in a similar manner in both plants, it then seems unlikely that ethylene biosynthesis was responsible for the epinasty observed in the space-grown seedlings.

Peroxidase is known to function in several biochemical reactions in plant tissues. Whether one specific pathway is preferentially affected by growth in balanced gravity still remains to be demonstrated conclusively. Certainly, continued study in this area is indicated by the findings obtained during the course of this program.

Another finding of interest is the increased utilization of two carbon fragments by seedlings growing on a clinostat over their erect controls. Both ethane and acetylene appear to have been metabolized more rapidly under these conditions (table 5). The significance of this finding and the ultimate fate of these compounds are still not clear. Because of the implications of altered metabolic processes during weightlessness, pursuit of this observation could prove to be of considerable interest in future space research.

The kinetic studies performed during this program (table 6) indicate that balanced gravity does not result in structural changes to the enzyme molecule which may affect its ability to catalyze biochemical reactions. The conclusions drawn from biochemical measurements, therefore, indicate that weightlessness does indeed influence specific physiologic and biochemical functions of the plant which are ultimately manifested by altered configurations of organs. These biochemical changes were not due to changes in the tertiary structure of the enzyme protein molecule, as some investigators had prophesied.

SUMMARY AND CONCLUSIONS

Profound effects of spaceflight have been demonstrated upon the biochemistry and physiology of wheat seedlings. These effects are, for the most part, duplicated on Earth by growth on the horizontal clinostat. The visible manifestations of growth in a balanced gravity environment (clinostat) are short curvature and root epinasty. Root growth is random when compared with the highly ordered pattern observed in control seedlings grown in the normal erect position. The growth rate does not appear to be affected, however.

The altered orientation of seedling organs is believed to be caused by the translocation of auxin and its concomitant biochemical reactions. The

TABLE 6. – Enzyme Kinetic Studies Using Lateral Roots of Wheat Seedlings Under 3 Conditions of Growth

Enzyme	K_m, M			V_{max}, mM/min./μg protein		
	Flight	Clinostat	Erect	Flight	Clinostat	Erect
Peroxidase	1.81×10^{-3}	2.86×10^{-3}	2.45×10^{-3}			
Glucose-6-phosphate dehydrogenase	4.00×10^{-5}	4.01×10^{-5}	4.02×10^{-5}	4.55×10^{-3}	4.37×10^{-3}	4.95×10^{-3}
Glyceraldehyde-3-phosphate dehydrogenase	2×10^{-3}	1.8×10^{-3}	1.8×10^{-3}	8.3×10^{-3}	7.2×10^{-3}	4.8×10^{-3}
Malic dehydrogenase	2.3×10^{-2}	2.3×10^{-2}	2.3×10^{-2}	1.1×10^{-2}	2.9×10^{-2}	7.7×10^{-2}
Transaminase	5.5×10^{-4}	2.9×10^{-4}	5.5×10^{-4}	1.0×10^{-2}	9.7×10^{-3}	8.9×10^{-3}
Cytochrome C reductase	4.3×10^{-6}	5.3×10^{-6}	2.8×10^{-6}	3.4×10^{-5}	6.9×10^{-5}	4.1×10^{-5}

biochemical parameters affected by growth in space are increased respiration, increased peroxidase activity at the site of epinastic curvature, and a possible influence upon carbohydrate metabolism. The latter effect may have resulted from the vibration incurred during lift-off or reentry.

Ethylene was not found in any of the seedling growth chambers. Since the sensitivity of these measurements is 0.1 ppb, it is doubtful that the compound was responsible for the altered orientation of seedling organs observed.

The physiologic effects observed were caused by alteration in the metabolism of the plant. Kinetic studies rule out the possibility of structural changes in the protein affecting the ability of the enzymes to catalyze biochemical reactions.

REFERENCES

ABLES, F. B. 1966. Auxin Stimulation of Ethylene Evolution. *Plant Physiol.*, **41**:585–588.

AKAZAWA, T.; AND CONN, E. E. 1958. Oxidation of Reduced Pyridine Nucleotides by Peroxidase. *J. Biol. Chem.*, **232**:403.

ANDREAE, W. A.; VENIS, M. A.; JURSIC, F.; AND DUMAS, T. 1968. Does Ethylene Mediate Root Growth Inhibition by Indole-3-Acetic Acid? *Plant Physiol.*, **43**:1375.

AURAND, L. W.; ROBERTS, W. M.; AND CARDWELL, J. T. 1965. A Method for the Estimation of Peroxidase Activity in Milk. *J. Dairy Sci.*, **39**:568–573.

BOYSEN-JENSEN, P. 1936. Uber die Verteilung des Wuchsstoffes in Keimstengein and Wurzein waehrend der Phototropischen und Geotropischen Krummung. K. Danske Vitenskab. *Selskab Biol. Meddelelser,* **13**:1–31.

CHADWICK, A. V.; AND BURG, S. P. 1967. An Explanation of the Inhibition of Root Growth Caused by Indole-3-Acetic Acid. *Plant Physiol.*, **42**:415–420.

CONRAD, H. M. 1968. Biochemical Changes in the Developing Wheat Seedling in the Weightless State. *BioScience,* **18**:645–652.

CONRAD, H. M.; AND YOKOYAMA, K. 1969. Response of Plants to Hypogravity Gradients. Proceedings of XI International Botanical Congress.

CONRAD, H. M.; AND YOKOYAMA, K. 1970. Epinastic Thresholds in a Hypogravity Gradient. *Physiol. Plantarum,* in press.

CROCKER, W.; ZIMMERMAN, P. W.; AND HITCHCOCK, A. E. 1932. Ethylene Induced Epinasty of Leaves and the Relation of Gravity to it. *Contrib. Boyce Thompson Inst.*, **4**:177–218.

CURTIS, C. R. 1967. Bean Rust Development in Relation to Gravity. *Phytopathology,* **57**:1025–1029.

DEDOLPH, R. R.; GORDON, S. A.; AND OEMICK, D. A. 1966. Geotropism in Simulated Low-Gravity Environments. *Am. J. Botany,* **53**:530–533.

DEDOLPH, R. R.; **WILSON, B. R.; CHORNEY**, W.; AND BREEN, J. J. 1966. Simulated Low-Gravity Environments and Respiration Metabolism in *Avena* Seedlings. *Plant Physiol.*, **41**:1520–1524.

GALSTON, A. W.; BONNER, J.; AND BAKER, R. S. 1953. Flavoprotein and Peroxidase as Components of Indoleacetic Acid Oxidase System of Peas. *Biochem. Biophys.*, **42**:456.

GALSTON, A. W.; AND DAVIES, P. J. 1969. Hormonal Regulation in Higher Plants. *Science,* **163**:1288–1297.

GASPAR, V.; AND LACOPPE, J. 1968. The Effect of CCC in AMO–1618 on Growth, Catalase, Peroxidase, and Indoleacetic Acid Activities in Young Barley Seedlings. *Physiol. Plantarum,* **21**:1104.

GILLESPIE-PICKARD, B.; AND THIMANN, K. V. 1966. Geotropic Response of Wheat Coleoptiles in Absence of Amyloplast Stark. *J. Gen. Physiol.*, **49**:1065–1086.

GRAY, S. W.; AND EDWARDS, B. F. 1968. The Effect of Weightlessness on Wheat Seedling Morphogenesis and Histochemistry. *BioScience,* **18**:638–645.

HALL, W. C.; AND MORGAN, P. W. 1964. Regulateurs Naturels del la Croissance Vegdule. Centre National de la Recherche Scientifique (Paris), pp. 727–745.

HENRY, R. J. 1963. Clinical Chemistry. Harper & Row (New York, N.Y.).

JOHANSEN, D. A. 1940. Plant Microtechniques. McGraw-Hill Book Co., Inc.

JOHNSON, S. P.; AND TIBBITTS, T. W. 1968. The Liminal Angle of a Plagiogeotropic Organ Under Weightlessness. *BioScience,* **18**:655–661.

KORNBERG, A.; AND HORECKER, B. L. 1962. Methods in Enzymology. Vol. I. Colowick and Kaplan, eds., Academic Press, Inc. (New York, N.Y.).

LINDERSTROM-LANG, K.; AND HOLTER, H. 1932. Contribution to the Histochemistry of Enzymes II. The Distribution of Peptidase in the Roots and Sprouts of Malt. Compt. rend. trav. lab. Carlsberg. *Ser. chim.,* **19**(4):1–19; *Z. physiol. Chem.,* **201**:9–30.

LINEWEAVER, H.; AND BURKE, D. 1934. Determination of Enzyme Dissociation Constants. *J. Am. Chem. Soc.,* **56**:658–666.

LYON, C. J. 1962. Gravity Factor for Auxin Transport. *Science,* **137**:432–433.

LYON, C. J.; AND YOKOYAMA, K. 1966. Orientation of Wheat Seedling Organs in Relation to Gravity. *Plant Physiol.*, **41**:1065–1073.

LYON, C. J. 1968. Wheat Seedling Growth in the Absence of Gravitational Force. *Plant Physiol.,* **43**:1002–1007.

MURPHY, J. B.; AND KIES, M. W. 1960. Spectrophotometric Determination of Protein in Dilute Solutions. *Biochim. Biophys. Acta,* **48**:382–384.

RACKER, E. 1965. Mechanisms in Bioenergetics. Academic Press, Inc. (New York, N.Y.).

RIDDLE, V. M.; AND MAZELIS, M. 1964. A Role of Peroxidase in the Biosynthesis of Auxin. *Nature,* **202**:391.

RUHLAND, W.; ET AL. 1962. Encyclopedia of Plant Physiology. Vol. 17, Part 2. Springer (Berlin).

SAUNDERS, B. C.; HOLMS-SIEDLE, A. G.; AND **STARK, B. F.** 1964. Peroxidase—The Properties and Uses of a Versatile Enzyme and of Some Related Catalysts. Butterworths (London).

SHEN-MILLER, J.; HINCHMAN, R.; AND GORDON, S. A. 1967. Thresholds for Georesponse to Acceleration in Gravity Compensated Avena Seedlings. *Plant Physiol.,* **43**:338–344.

SHOLANDER, P. F. 1947. Analyzer for Accurate Estimation of Respiratory Gases in One Half Cubic Centimeter Samples. *J. Biol. Chem.,* **167**:235–250.

SIEGEL, S. M. 1955. The Biochemistry of Lignin Formation. *Physiol. Plantarum,* **8**:20.

SIEGEL, L.; AND ENGLAND, S. 1961. Beefheart Malic Dehydrogenase I. Properties of the Enzyme Purified From Extracts of Acetone Dried Powders. *Biochim. Biophys. Acta.,* **54**:67–76.

STEPHENS, E. R.; AND BURLESON, F. R. 1967. Analysis of the Atmosphere for Light Hydrocarbons. *APCA J.*, **17**:147–153.

SZYMANSKI, H. A. 1964. Biomedical Applications of Gas Chromatography. Plenum Press (New York, N.Y.).